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# Nitrogen plasma surface modification enhances cellular compatibility of aluminosilicate glass

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## Abstract

The effect of Active Screen Plasma Nitriding (ASPN) treatment on the surface-cellular compatibility of an inert aluminosilicate glass surface has been investigated. ASPN is a novel surface engineering technique, the main advantage of which is the capacity to treat homogeneously all kind of materials surfaces of any shape. A conventional direct current nitriding unit has been used together with an active screen experimental arrangement. The material that was treated was an ionomer glass of the composition  $4.5\text{SiO}_2\text{-}3\text{Al}_2\text{O}_3\text{-}1.5\text{P}_2\text{O}_5\text{-}3\text{CaO-}2\text{CaF}_2$ . The modified glass surface showed increased hardness and elastic modulus, decreased surface roughness. The incorporation of nitrogen-containing groups was confirmed using X-ray photoelectron spectroscopy. The modified surface favoured attachment and proliferation of NIH 3T3 fibroblasts.

## Keywords

Active screen plasma nitriding; fibroblast; glass; ionomer; surface

## 1. Introduction

Surface modification is employed to alter the surface characteristics of materials while maintaining the bulk properties [1-3]. Surface modification of biomaterials is often required to alter those characteristics that play a role in their interaction with the biological environment [4-7]. Plasma treatment modifies the surface properties of materials [8] resulting in changes extending from a few nanometres to  $\sim 10\text{ }\mu\text{m}$  without affecting the bulk properties of the material [9], surface chemistry can be selectively controlled and a variety of chemical structures can be produced [10]. Plasma treatment affords control over wettability, chemical inertness, hardness and biocompatibility [11-12]. Plasma-modified materials exhibit amended response characteristics when in contact with the biological environment [13]. Active Screen Plasma Nitriding (ASPN) is a surface engineering method historically used to modify metallic surfaces such as stainless steel, chromium and titanium by introducing nitrogen into the surface improving the surface microhardness, wear and corrosion resistance, microstructure and morphology [14-17]. ASPN offers reduced gas and energy consumption, minimisation of pollutant emission, and short treatment times. Despite the fact that conventional direct current plasma nitriding has been proved efficient in the treatment of simple shapes and small loads, there exist many difficulties during the treatment; these include (i) maintaining uniform chamber temperature, (ii) arcing, and (iii) non-uniform appearance at edges [18-22]. Conversely, ASPN is a relatively novel surface modification that allows the homogeneous treatment of surfaces of any morphology [23].

Ionomer glasses based on the general composition  $\text{SiO}_2\text{-Al}_2\text{O}_3\text{-P}_2\text{O}_5\text{-CaO-CaF}_2$  are used glass ionomer cements in dentistry [24]. Recent developments led to glass compositions that offer radiopacity, translucency, controlled setting reaction, as well as release of therapeutic ions such as fluorine and strontium [25-28]. The glass composition used in this study,  $4.5\text{SiO}_2\text{-}3\text{Al}_2\text{O}_3\text{-}1.5\text{P}_2\text{O}_5\text{-}3\text{CaO-}2\text{CaF}_2$ , has been extensively characterised and both its structure and crystallisation mechanism are well

understood [29-31]. Plasma surface modification on a glass surface (without the addition of coatings) in order to study the cellular compatibility of the modified surface has not previously been conducted. Therefore the aim of this work is to investigate the effect of ASPN on the surface properties of an inert glass composition as well as the effect of the treatment on fibroblast/surface interactions.

## 2. Materials and methods

The glass composition  $4.5\text{SiO}_2\text{-}3\text{Al}_2\text{O}_3\text{-}1.5\text{P}_2\text{O}_5\text{-}3\text{CaO-}2\text{CaF}_2$  was produced by a melt quench route that has been previously reported in detail [29]. Cylindrical samples (diameter 15 mm, length 20 mm) were cast and polished following this method. ASPN surface modification has been described previously by Li *et al.* [32]. Prior to treatment all samples were cleaned with distilled water and ethanol. Samples were ASPN treated at 400 °C in a gas mixture of 25%  $\text{N}_2$  and 75%  $\text{H}_2$  for 1 h; the plasma chamber pressure was 2.5 mbar, the voltage was 250V and the current was 1 A. After treatment all samples were stored under vacuum ( $10^{-2}$  mbar). The hardness and elastic modulus of the surfaces were measured before and after treatment by nanoindentation. The hardness and elastic modulus were calculated from the mean of six measurements. The equipment used was a Nano Test 600 machine (Micro Materials UK). Surface topography was measured before and after treatment using a MicroXAM interferometer (Scantron, UK) operating a white light source. Scanning Probe Image Processor software (Image Metrology, Denmark) was employed for the analysis of the acquired images, yielding average roughness ( $S_a$ ) and root-mean-square roughness ( $S_q$ ) values, which were the mean of five measurements at separate locations. Scan areas sufficiently large as to be representative of the overall surface of the sample were identified. X-ray photoelectron spectroscopy (XPS) was performed to examine any chemical changes introduced on the surface of the treated glass. A bespoke XPS was used for the analysis of both treated and untreated samples. The software used was produced by PSP Ltd, UK. The pass energy was 50 eV and the X-ray gun operated at 10 keV. Spectra were acquired over the binding energy range -10 to 1,200 eV. The vacuum pressure in the analysis chamber was less than  $10^{-8}$  mbar.

All chemicals for the cell culture studies were purchased from Sigma-Aldrich UK. NIH 3T3 fibroblasts were used to study the cellular compatibility of the treated and untreated glass surfaces. Prior to cell seeding, the treated and untreated glass surfaces were autoclaved for 15 minutes at 120 °C. 3T3 fibroblasts were cultured for a week using the standard 3T3 protocol. Cells were grown in S-DMEM (Supplemented Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS, 2.4% L-glutamine, 2.4% HEPES buffer and 1% penicillin/streptomycin, and fed every second day during the cell culture. All sterilised glass samples were placed in 12 well-plates, seeded at a cell density of  $1.2 \times 10^6$  cells/sample and incubated at 37 °C at 5%  $\text{CO}_2$  and 100% relative humidity for 4 days. The cell seeded surfaces were examined using SEM (JSM 6060 LV, JEOL, Oxford Instruments Inca, UK). The operating voltage was 10 kV whereas the working distance was 10 mm and the spot size was 3 nm. Prior to testing, the cell seeded samples were chemically fixed following a standard protocol described previously in detail [33]. The fixed cell seeded samples were Pt coated by sputtering at 25 mA and 1.5 kV. The thickness of the Pt coating was 10-12 nm.

## 3. Results and Discussion

Table 1 shows the nanoindentation results of the plasma treated and untreated glass. The hardness and elastic modulus were clearly increased after the treatment. According to the literature, plasma treatment has been used in the past in order to improve the mechanical properties of glasses, ceramics and polymers [34-37]. Schrimph *et al.* reported that the incorporation of nitrogen into silicate melts increased the hardness, refractive index, chemical durability and glass transition temperature ( $T_g$ ) proportionally to increasing nitrogen content [38]. Grande *et al.* also reported that quenching of fused mixtures of nitrides and oxides or reacting  $\text{NH}_3$  with molten oxides on the surface of silicate and phosphate glasses resulted in an increase of the surface hardness. This was explained based on the assumption that only one of the four divalent oxygen atoms surrounding the glass formers, in this case silicon and phosphorus, could be replaced by trivalent nitrogen resulting in a more tightly linked glass

network [39]. In our case there is a significant increase in the hardness and elastic modulus after the ASPN treatment; these results are shown in Table 1. As the treatment temperature was well below the glass transition temperature of the glass (around 670 °C) as reported previously [40], at 400 °C there is a possibility that annealing effects may have resulted in the incorporation of trivalent nitrogen in the glass network, having the effect of crosslinking further the glass network at the surface and therefore increasing hardness and elastic modulus. Further investigation however should be conducted in order to establish the hardening mechanism.

Figures 1(a) and (b) show the surface topography of both treated and untreated glass samples. The  $S_a$  and  $S_q$  for the untreated glass are 96.8 nm and 121.0 nm respectively, while for the treated glass surface the  $S_a$  and  $S_q$  were 66.2 nm and 89.5 nm respectively; these values demonstrate that ASPN treatment significantly decreased the surface roughness. Furthermore, some surface porosity was observed on the surface of the ASPN treated glass exhibiting depths > 200 nm below the sample surface, as shown in Figure 1(c). XPS measurements of the treated and untreated glass samples revealed the presence of nitrogen containing species on the plasma modified glass surface indicated by the presence of a broad peak at a binding energy of 403 eV, associated with N 1s photoelectrons, as shown in Figure 1(d). The untreated glass surface did not exhibit a peak at this binding energy. The nitrogen content of the treated glass surface was found to be 1.5% of the total elemental composition. The nitrogen-containing functional groups likely consist of amine (-NH<sub>2</sub>) moieties, given that the plasma composition is 25 % N<sub>2</sub> and 75 % H<sub>2</sub>. The amine groups are likely to be attached to the surface via Si-N bonds. The treated and untreated ionomer glass samples were also subjected to cell culture studies. The purpose of this preliminary study was to observe the cellular response to ASPN treatment. Figures 2(a)-(c) are SEM images showing the absence of 3T3 fibroblasts after their seeding on the surface of untreated glass, following a 4 day incubation period. In contrast Figures 2(d)-(f) show the presence of 3T3 fibroblasts seeded on the ASPN treated glass, following a 4 day incubation period. This cellular response is in good agreement with previous work by Freeman *et al.* [41] wherein an animal study of the same glass composition did not integrate well with bone. Scar tissue formation was observed at the interface of the glass with the native tissue, showing that the glass surface was not favourable to cellular compatibility. On the surface of the ASPN treated glass studied in our work, it is clear that the fibroblasts are attached to the sample surface, indicated by the presence of cytoplasmic projections. The cells were not only interconnected but they also exhibited the appropriate stellate shape. In addition, the cytoplasmic extensions and the filopodia of the adjacent cells were clearly conjoined. *In vitro* cell attachment on material surfaces is usually mediated by glycoproteins such as fibronectin (Fn) and vitronectin (Vn).

The attachment of cells to surfaces depends on the substrate mechanical properties, surface topography, and specifically on the substrate chemistry that controls the nature of the protein layer [42]. It has been reported that plasma treatments enhance cell attachment and proliferation due to the presence of new functional groups on the surface that results in enhanced activity of glycoproteins [43-48]. For example, it has been reported that nitrogen plasma treated polymeric surfaces can develop such adsorptive characteristics to attract both Fn and Vn glycoproteins, suggesting that the presence of amine groups can result in cell attachment enhancement [49-50]. Previous work by Kaklamani *et al.* showed that a similar ASPN treatment of ultra-high molecular weight polyethylene (UHMWPE) surfaces promoted fibroblasts attachment and proliferation, while cells did not attach on the untreated UHMWPE surfaces [33]. Cells exhibit great sensitivity to substrates with specific nano- and micro-scale topographies [51-52]. Cells are also able to react to the surface mechanical properties. A mechanical balance should be maintained between the cells and the environment they grow in, for tissue formation, cohesion, homeostasis and signalling between the cells and the substrate [53]. Hence, cellular behaviour depends on the mechanical properties of the tissue substrate they are attached to [54]. In the work presented here, the plasma treatment has little effect on the surface topography. The surface exhibits many orders of magnitude greater modulus than cells, although the hardness and Young's modulus of the surface are increased further by plasma treatment. However it is not

anticipated that this will affect cell attachment. It is likely that the change in surface chemistry, specifically the incorporation of nitrogen-containing groups such as amine moieties, which enhances the adhesion of fibroblasts.

Generally, a study on the attachment of cells on a plasma modified surface has not been reported and there is minimal literature available in this area. Considering the simplicity of the plasma treatment, as well as the advantage of a homogeneous surface treatment, makes this technique extremely attractive and further study in the area of cellular activity could lead to the technique reaching its full potential in this area of research.

#### 4. Conclusions

The surface of an ionomer glass composition was subjected to ASPN treatment for 1 h at 400 °C in a gas mixture of 25% N<sub>2</sub> and 75% H<sub>2</sub>. The treatment resulted in:

1. Increased hardness and elastic modulus of the glass surface.
2. Decreased surface roughness.
3. Incorporation of nitrogen containing chemical species on the glass surface.
4. Greatly enhanced surface biocompatibility and adhesion of 3T3 fibroblasts.

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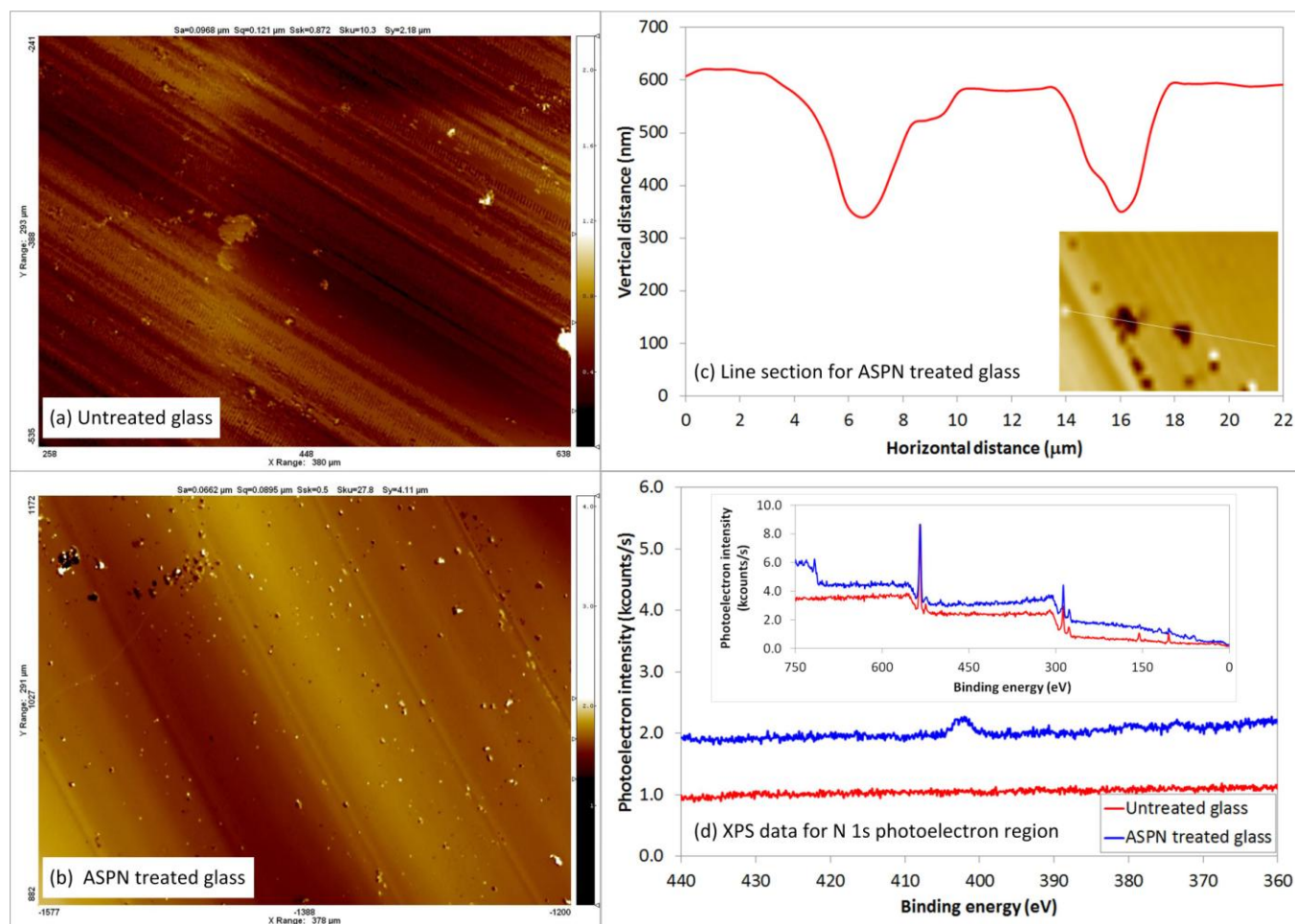
## Tables

**Table 1:** Hardness and elastic modulus of untreated and ASPN treated glasses.

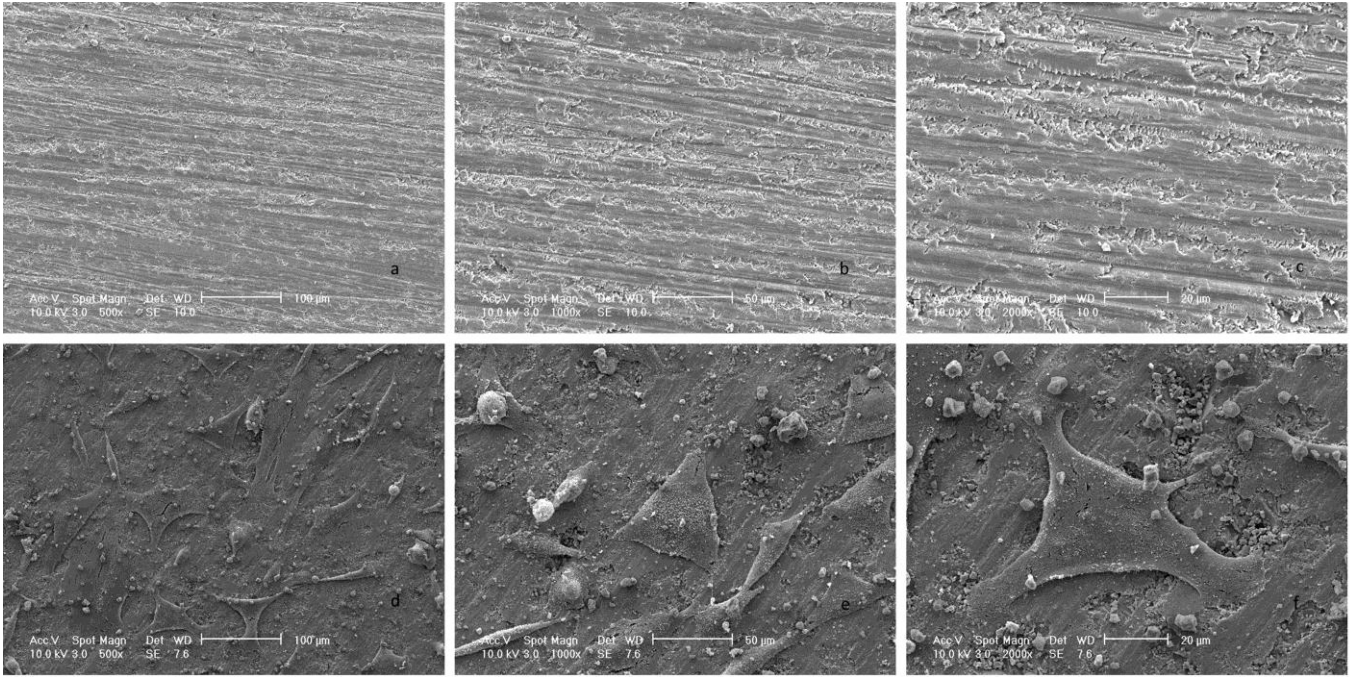
Material	Hardness (GPa)	Elastic modulus (GPa)
Untreated glass	8.3 ( $\pm 0.3$ )	106.5 ( $\pm 2.0$ )
ASPN treated glass	16.5 ( $\pm 2.4$ )	177.8 ( $\pm 5.9$ )



## Figures



**Figure 1:** 2D topography of (a) untreated and (b) ASPN treated glass surfaces, (c) line section of pores on the ASPN treated glass surface, (d) XPS spectra for the N 1s photoelectron binding energy range.



**Figure 2:** SEM images of the untreated glass surfaces 4 days after seeding with 3T3 fibroblasts at magnifications (a) x500, (b) x1000 and (c) x2000; SEM images of ASPN treated glass surfaces seeded with 3T3 fibroblasts at magnifications (d) x500, (e) x1000 and (f) x2000.